Laboratory study on the impact of pH and salinity on the fluorescence signal of Natural Organic Matter (NOM) relevant to groundwaters from a Canadian Shield sampling site
Vanessa Borraro, Rémi Riopel, François Caron and Stefan Siemann

ABSTRACT
Fluorescence spectroscopy with the spectral resolution routine PARAFAC is a leading tool to analyze Natural Organic Matter (NOM) in waters. This routine resolves spectra into humic-, fulvic- and protein-like components, which helps interpret the NOM dynamics in environmental systems. This work is one of the first systematic studies dealing with the impact of chemical perturbations on the fluorescence spectral interpretation of NOM. The samples, taken at two Canadian Shield locations (a shallow set and a deep set to \(-650\) m), were perturbed for pH (‘titrations’ from pH 4 to 10) and salinity (from \(\sim 0.02\) to \(3\%\) salt content), then analyzed by fluorescence/PARAFAC. Our fluorescence signals for the three components showed no clear change with pH, as would be expected with a classic titration. The signals were reproducible between replicates for the humic- and protein-like components, but less so for the fulvic-like components. Changes of salinity only had a small impact on the fluorescence signal (a \(\sim 2.7–3.4\%\) signal decrease for each salinity unit, \(\%\)) for the three components in this salinity range.

Key words | fulvic material, groundwater, humic material, Natural Organic Matter (NOM)

INTRODUCTION
Natural Organic Matter (NOM) is present in all natural waters; NOM is a collection of molecules and fragments originating from the breakdown of biomass, for example, carbohydrate residues (cellulose, lignin, etc.), plant residues (chloroplasts, etc.), lipids, fats, proteins, etc. These organic molecules or fragments are ubiquitous; the term given to the NOM from these processes is primary, terrestrial (terrestrial) or allochtonous (Morel & Hering 1993; Leenheer & Croué 2005). In turn, exudates from biological activities, breakdown of dead biomass and residues from the build-up or recycling of biomass constitute secondary NOM. The latter type of NOM is often termed autochtonous (Morel & Hering 1993; Leenheer & Croué 2003) to reflect the biogeochemical recycling of carbon. This recycling has been reported in lakes (Fellman et al. 2010), in carbon-deprived environments such as the open sea (Coble 1996; Kowalczuk et al. 2005) or deep groundwaters (Caron et al. 2010). NOM has several environmental roles, among which our broad focus includes acid-base control of natural waters (Litaor & Thurman 1988; Chapman et al. 2008). Other types of impacts include transport and speciation changes of radiocontaminants (Killey et al. 1984; Penrose et al. 1990; Marley et al. 1993; Schmitt et al. 2003; Caron & Mankarios 2004; Caron et al. 2007), and changes in metal speciation, transport and toxicity (Smith & Kramer 2000; Vigneault et al. 2000; Daughney et al. 1999; Smith et al. 2004; Galvez et al. 2008).

The NOM constituents contain various functional groups of which the carboxylic and phenolic acid groups are well established (Litaor & Thurman 1988; Morel & Hering 1993; Stumm & Morgan 1996; Frimmel 1998; Ritchie & Perdue 2005). Other weak acid–base groups have been reported in the mid pH range, e.g. 5.1–7.5 and...
7.6–9.2 (Smith & Kramer 1999). These could include sulfur-based groups (Smith et al. 2004), phosphate-based and other carboxylic groups on cell membranes (Daughney et al. 1999). It is expected that NOM constituents exhibit acid–base and metal-binding properties similar to discrete functional groups. These groups, in turn, are expected to behave similarly to separate functional groups with a range of similar properties, and also dissimilarities because of the immediate molecular environments surrounding the acid–base groups.

Historically, humic substances have been characterized into three broad classes using wet acid–base fractionation: (1) humic acid, (2) fulvic acid and (3) humin (Morel & Hering 1993; Stumm & Morgan 1996). Other wet schemes have been developed, such as column separations (Leenheer 1981; Leenheer & Croué 2003). The advantage of wet methods rests with the separation of the NOM constituents, based mostly on chemical behavior, but the process is invasive and the manipulations can be tedious and lengthy. Fluorescence spectroscopy emerged as a leading alternative to characterize NOM (Coble 1996; Stedmon et al. 2005; Stedmon & Markager 2005; Holbrook et al. 2006; Fellman et al. 2010). The method is fast, non-destructive and non-invasive. This method tracks the optical characteristics of the NOM constituents in an Excitation–Emission Matrix (EEM), which can be numerically decomposed into individual and independent components using computational routines such as Parallel Factor Analysis (PARAFAC; see Andersen & Bro (2003)) or Fluorescence Regional Ingestion (FRI; see Chen et al. (2003)). These routines have resolved the NOM into several components, among which the humic-like, fulvic-like and protein-like components have been reported in most studies (Coble 1996; Mounier et al. 1999; Chen et al. 2005; Caron et al. 2010; Caron & Smith 2011). A drawback of the fluorescence technique is the lack of a direct reference to a chemical behavior for the material analyzed. For example, the overall similarity of humic-like fluorescence spectra to those of humic extracts (based on XAD resin extractions) has been mentioned by Coble (1996). The same author has also noted similarities between spectra of melanoidins (a potential model compound for humic substances) and humic-like spectra. The protein-like region is more straightforward, as it relates to the tyrosine and tryptophan residues in proteins (Coble 1996; Chen et al. 2003).

Several parameters can change the fluorescence response of NOM: for example, the impact of pH on NOM fluorescence is not well known, or it is limited to just a few pH values (Mobed et al. 1996; Baker et al. 2007). Changes in signal intensity with salinity have been reported (Coble 1996; Kowalczuk et al. 2005). It is important to note, however, that these authors have developed empirical relationships between fluorescence signal and salinity, without taking into account that the composition of the NOM was different between the samples. The presence of metals is another example of a parameter that can alter fluorescence signals (Smith & Kramer 2000; Bai et al. 2008).

Our study addresses limitations related to pH and salinity changes (investigations with metals are outside the scope of this work). In this systematic study, we are using consistent sets of data by purposefully perturbing only the sample matrix (i.e. through changes in salinity and pH), thus eliminating sample-to-sample variations.

The objective of this work is to investigate the changes in NOM fluorescence signals from controlled perturbations, relevant to environmental systems: the first impact is related to pH changes (proton titration) of three sets of samples, and the second impact consists of changing the salinity of one sample set. A novel aspect of this work is the separation of the fluorescence response into individual components using the PARAFAC routine. This provides additional insights into the impact of chemical perturbations on individual fluorescing components.

**METHODOLOGY**

**Overview of the experimental approach**

In the first part of this work, fluorescence signals were recorded following pH titrations from pH 4–10, which bracket environmental conditions found at a boreal forested site on the Canadian Shield (CS). In the second part of this work, salinity changes were applied to cover two orders of magnitude, from ~0.02 to ~3‰ (i.e. ionic strength of $10^{-3.47}$–$10^{-1.31}$ mol/L as NaCl). This salinity range corresponds to actual samples in our previous work (Caron et al. 2010). These changes are environmentally relevant under human or natural impacts, such as the mixing of...
deep and shallow groundwaters (high salt content to low salt content), or the mixing between types of shallow groundwaters (pH and salinity changes, human impacts such as landfills).

Samples and salicylic acid standard

Archived samples from two previous sampling campaigns were used in this work (Table 1). Samples from shallow groundwaters were taken from three stations near a former liquid dispersal area (LDA) at the Chalk River Laboratories (CRL – see Caron & Mankarios (2004)). The deep groundwater samples originated from an (undisclosed) pristine location on the CS, approximately 200 km west of Ottawa, ON. All these samples were kept in a pre-cleaned 40 mL amber glass bottle (with Teflon-backed stopper, I-Chem) stored in a refrigerator at approximately 4°C.

Salicylic acid (SA) standards (Fisher Chemicals) were prepared in deionized (Millipore) water. Solutions of concentrations $10^{-5}$ M (titrations) or $10^{-6}$ M (fluorescence) were used and freshly prepared on a weekly basis. Fresh deionized water (Millipore) was used as blanks.

Titrations

Titrations were performed using a Metrohm 785 DMP Titrisol automatic titrator, controlled by the Titrisol Workcell software (Brinkmann™) and a Metrohm glass electrode, calibrated every day of use. The titrants were $1.000 \times 10^{-1}$ M HCl and $1.254 \times 10^{-3}$ M NaOH (Fisher Chemicals), both calibrated in-house. Each SA standard and sample was split into two aliquots, a sacrificial aliquot and an electrodeless aliquot, because the pH electrode created a dominant artifact in the fluorescence signal at high pH (Caron, unpublished). Both the SA standards and samples were treated similarly.

The sacrificial aliquot consisted of first titrating the SA standard or sample (100 mL per replicate, in triplicate) to pH 4.0 to remove carbonates, followed by a base titration to pH 10. The base titration was programmed to stop at pH 4.5, 5.0, 5.5, 6.5, 7.5, 8.0, 9.0 and 10.0, to bracket the expected $pK_a$’s of the SA and the NOM. At each stop point, a 10 mL aliquot was removed and discarded, as it would be for fluorescence analysis. The titrant volumes at these stop points were recorded for the electrodeless aliquots. All of these aliquots were discarded.

The electrodeless aliquots (also 100 mL per replicate, in triplicate) were titrated similarly to the above, except that no pH electrode was allowed to contact the solution. The volumes of titrant (acid or base) were pre-programmed with the software to bring the solutions to the same stop points as above. At each stop point, a 10 mL aliquot was withdrawn for fluorescence analysis; the pH was measured afterwards for confirmation. These were the pH values reported therein. The pH variation between replicates was ± 0.15 pH unit.

Salinity perturbations

Two sets of salinity perturbations were performed. The first set was done on shallow groundwaters from station LDA 22 to prepare an empirical calibration. Seven salinity points, from 0.02 to 2.85 parts per thousands (ppt, ‰), were selected based on the salinity values in the deep CS borehole (King-Sharp, unpublished). For each of the three replicates, 9 mL of LDA sample was mixed with 1 mL salt adjuster (deionized water plus concentrated NaCl, to match the

<table>
<thead>
<tr>
<th>Sampling station type</th>
<th>Note and description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDA 3</td>
<td>Clean and uncontaminated, ~3 m below surface. Fraction analyzed: 5 kDa filtrate.</td>
</tr>
<tr>
<td>LDA 22, LDA 23</td>
<td>Contaminated groundwater in the flowpath of LDA 3. Stations are located ~50 m downstream from a former liquid dispersal area (LDA), no longer in use. The LDA is open to the atmosphere and wet depositions contact the residual radionuclides to the groundwaters. Fractions analyzed: 5 kDa retentates, because they contain the majority of the $^{241}$Am and $^{137}$Cs.</td>
</tr>
<tr>
<td>Deep groundwater</td>
<td>Reference: Caron et al. (2010)</td>
</tr>
<tr>
<td>CS</td>
<td>Canadian Shield, pristine and uncontaminated; Interval no. and nominal depth below surface (in brackets): Int. 2 (108 m), Int. 3 (139 m), Int. 5 (285 m), Int. 8 (503 m), Int. 11 (620 m) and Int. 12 (650 m). Fractions analyzed: 0.45 μm filtrates.</td>
</tr>
</tbody>
</table>
measured salinity). Each salinity-adjusted sample was then measured by fluorometry and the signals were resolved into three components using PARAFAC.

The second set was performed on the CS samples. One original and one salt-perturbed fluorescence measurement was done on each sample. Each salt-perturbed replicate was adjusted to 2.85‰ by adding 1 mL of salt adjuster (deionized water containing the appropriate amount of concentrated NaCl) to 9 mL of original sample.

Fluorescence spectroscopy and spectral resolution

Spectroscopy

The fluorescence spectrophotometer was an Olis RSM 1000 F1 (Bogart, GA) equipped with an excitation source (150 W xenon arc lamp) and a DeSa monochromator/ photon counter. All the modules were controlled with the Olis GlobalWorks software. The excitation scans were increased from 200 to 450 nm at 10 nm intervals (10 nm bandpass), whereas emissions were measured from 250 to 600 nm (20 nm bandpass), using a dual-grating monochromator (600 lines/mm each grating). The samples were measured in a 1 cm quartz cuvette (Starna Cells, Atascadero, CA), at a controlled temperature of 20°C. The integration time was set at 0.1 sec on the software.

The fluorescence data were saved as 3D graphic files and in Excel spreadsheets. The spreadsheets were processed using an in-house program in a MATLAB platform (Smith, unpublished) to remove the Rayleigh/Tyndall scattering lines and the Raman peak, and to generate EEMs. The EEM, in turn, were resolved using a PARAFAC routine (Andersen & Bro 2003), also in MATLAB (Caron et al. 2010; Caron & Smith 2011). The samples were pooled for each type of perturbation (pH, salinity) and run as standalone sets. For each pool (pH or salinity), a routine was run with all the files, or sample-by-sample, similar to the split-half analysis (Stedmon & Bro 2008) to achieve the highest possible confidence level. Based on our past experience (Caron & Smith 2011 and references therein), the number of components was set at three: humic-like, fulvic-like and protein-like components. This choice of components was deliberate for comparison with prior work (Coble 1996; Stedmon et al. 2003; Stedmon & Markager 2005; Holbrook et al. 2006).

The humic-like component consisted of a broad region in the high emission wavelength range (400–600 nm), with a low to moderate fluorescence intensity. The fulvic-like component had a blue-shifted emission range and occupied a smaller area than the humic-like region. The protein-like component often had the most intense emission but a small area at an excitation wavelength of ~280–300 nm and an emission wavelength of 300–350 nm. It is noted that the terms ‘humic’-, ‘fulvic’- or ‘protein-like’ may or may not imply the chemical structure and behavior of their respective names. These nominal terms are based solely on fluorescence activity. The intensity signals are reported in triplicate unless otherwise mentioned, which means a combination of separate titrations, fluorescence measurements and PARAFAC processing.

Standards and benchmarking

Each set of samples was measured along with fresh deionized water and freshly prepared 10^-6 M SA standard (<1 week old). The SA signal intensities were reproducible within ±2% during the experimental period. Earlier analyses of archived shallow groundwaters (LDA 22), used as benchmarks, gave reproducible intensities within ±3.1% for the humic-like, ±5.0% for the fulvic-like and ±4.3% for the protein-like components (five replicates, three components each; unpublished).

RESULTS AND DISCUSSION

Fluorescence signal changes with pH (titrations)

All titration curves for the sacrificial aliquots of SA, controlled with a pH electrode, showed the expected S-shaped trend. Similarly, the samples showed the expected ‘flattened’ titration, typical of weak polyprotic acids (titrations not shown).

The titration showing the fluorescence intensity of the SA standard is shown in Figure 1. The standard deviation of the signal intensity (n = 3) was 0.3–1.6%. There was no convincing change of the signal intensity vs pH, albeit the value at pH ~ 10 was higher by ~4% compared to the signal at other pH values. The absence of signal change is
not surprising, as the pHs of these perturbations are between the first (∼2.9) and second pKₐ (∼13.2) of SA (Martell & Smith 1977). Only small changes in deprotonation are expected between pH ∼5–10, as these values are too far from the pKₐs to affect the other SA species. No shift in the excitation/emission (Ex/Em) maximum was observed (e.g. for Ex = 300 nm, Em was always 402 ± 2 nm).

The fluorescence titration curves, resolved into components by PARAFAC, are shown in Figures 2(a)–(c) for the clean (LDA 3) and contaminated shallow groundwaters (LDA 22 and 23). Even though the pH range covered at least partially the dissociation range of the carboxylic and phenolic groups, other expected groups, e.g. phosphates, sulfides and amines, would also be affected in this pH range.

There was no convincing trend of the signal with pH for any of the humic-, fulvic- and protein-like components. The intensities were generally reproducible for the humic-like and protein-like replicates for any of the LDA samples; however, the signals of the fulvic-like components were not consistent or reproducible between replicates for all three samples, especially for LDA 22 (Figure 2(b)). It is conceivable that the protonation and deprotonation from the titration induced changes in conformation in the NOM constituents (De Haan et al. 1987; Mobed et al. 1996), similarly to the coiling and uncoiling of proteins. Changes in conformation could affect the exposure of functional groups to water (Mobed et al. 1996), or the fluorescent moieties might not be in immediate contact with the solvent, as for undegraded proteins (Burstein et al. 1975). In either case, a pH change might not affect fluorescence. It is also possible that pH-induced spectral shifts (Mobed et al. 1996) could change the intensities reported by PARAFAC and misreport a component (e.g. fulvic-like). This is unlikely in our work, as a shift in the fulvic-like signal (which could have caused the non-reproducible signals) could artificially over-report the signal of the humic-like component and increase the variability of the latter. The high variability of the fulvic-like signal is still contentious at the moment.

Changes of fluorescence with salinity

Figure 3 shows the signal variations of the components as a function of salinity (in NaCl mass units) for our benchmark sample (LDA 22). There was a small, but noticeable, signal decrease for the three components, with an increase of salinity. This is not surprising, as ions are known quenchers (Lakowicz 2006). This variation with salinity was linear with a correlation of $r^2 = \sim 0.74$ to ∼0.91 for the three components. The slopes extracted from the plot correspond to a fluorescence signal decrease of 2.7% for each salinity unit (expressed in parts per thousand as NaCl, ‰) for the
The fulvic-like component, 3.4% for each salinity unit for the humic-like and 3.4% for each salinity unit for the protein-like component. It is conceivable that salinity-induced coil-\underbrace{ing\,\text{and}\,uncoiling\,of\,NOM\,constituents}, which could also impact the electrostatic environments at the molecular level, might affect the fluorescence signals. The molecular origin of the slope difference for the fluorescence components was not specifically investigated.

There is only a small body of evidence available to predict the changes in fluorescence intensity with salinity (Kowalczuk et al. 2005). Earlier relationships have been proposed (Coble 1996; Kowalczuk et al. 2005), which correspond to a signal decrease of \(\sim 4\%\)–8% in humic-like fluorescence per salinity unit (in parts per thousand, %‰). Our empirical relationship (Figure 3) suggests that the signal decrease is slightly lower than this, e.g. fulvic-like (2.7% fluorescence intensity per salinity unit), humic-like and protein-like (3.4% per salinity unit each), for up to almost 5%‰ salinity. Our empirical relationship is an improvement over the previous models; it originated from one set of data, with a systematic approach. Separate relationships were obtained for the three components, which is not available elsewhere, to the author’s knowledge.

The model developed above was applied to deep CS samples (Table 2). The table shows original measurements (item 1 in the columns), the measurements in high salinity (item 2), purposefully adjusted to the maximum salinity found in the highest CS sample, and (item 3) the predicted value from the model. It is understood that the model is empirical and it was determined using a surface NOM, as opposed to deep water NOM. Nevertheless, the model gives a reasonable prediction (item 4) for the separate components. This confirms that the salinity has only a small impact on fluorescence measurements.

**CONCLUSIONS**

This work is among the first systematic studies showing the impact of sample disturbances such as pH and salinity changes on the fluorescence signals of NOM. The study has also answered the question about the impact of these changes on separate signals of humic-, fulvic- and protein-like components. We found no convincing change of fluorescence with pH for any of the fluorescence components of the NOM. We have reported, however, that titrations yielded inconsistent signals between replicates for the fulvic-like components. It is likely that pH changes have induced a conformational change in the NOM constituents, affecting the fluorescence signals, but only to some extent.
Table 2 | Corrections for salinity using the empirical model shown in Figure 3

<table>
<thead>
<tr>
<th>Sample interval no. (original salinity)</th>
<th>Component</th>
<th>1. Original measurement</th>
<th>2. Salt-adjusted (constant salinity)</th>
<th>3. Calculated&lt;sup&gt;a&lt;/sup&gt;</th>
<th>4. Signal difference&lt;sup&gt;b&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (salinity = 0.1‰)</td>
<td>Humic-like</td>
<td>2.06</td>
<td>1.94</td>
<td>1.91</td>
<td>-1.4</td>
</tr>
<tr>
<td></td>
<td>Fulvic-like</td>
<td>2.25</td>
<td>2.12</td>
<td>2.08</td>
<td>-1.6</td>
</tr>
<tr>
<td></td>
<td>Protein-like</td>
<td>0.76</td>
<td>0.76</td>
<td>0.71</td>
<td>-5.9</td>
</tr>
<tr>
<td>3 (salinity = 0.02‰)</td>
<td>Humic-like</td>
<td>1.89</td>
<td>1.80</td>
<td>1.77</td>
<td>-1.5</td>
</tr>
<tr>
<td></td>
<td>Fulvic-like</td>
<td>2.04</td>
<td>1.97</td>
<td>1.89</td>
<td>-4.3</td>
</tr>
<tr>
<td></td>
<td>Protein-like</td>
<td>0.92</td>
<td>0.69</td>
<td>0.87</td>
<td>25.8</td>
</tr>
<tr>
<td>5 (salinity = 0.05‰)</td>
<td>Humic-like</td>
<td>1.60</td>
<td>1.57</td>
<td>1.51</td>
<td>-4.0</td>
</tr>
<tr>
<td></td>
<td>Fulvic-like</td>
<td>1.86</td>
<td>1.84</td>
<td>1.72</td>
<td>-6.8</td>
</tr>
<tr>
<td></td>
<td>Protein-like</td>
<td>0.65</td>
<td>0.61</td>
<td>0.62</td>
<td>1.2</td>
</tr>
<tr>
<td>8 (salinity = 0.35‰)</td>
<td>Humic-like</td>
<td>0.83</td>
<td>0.80</td>
<td>0.80</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Fulvic-like</td>
<td>1.28</td>
<td>1.30</td>
<td>1.20</td>
<td>-7.8</td>
</tr>
<tr>
<td></td>
<td>Protein-like</td>
<td>0.66</td>
<td>0.79</td>
<td>0.65</td>
<td>-18.4</td>
</tr>
<tr>
<td>11 (salinity = 1.79‰)</td>
<td>Humic-like</td>
<td>0.14</td>
<td>0.15</td>
<td>0.14</td>
<td>-2.7</td>
</tr>
<tr>
<td></td>
<td>Fulvic-like</td>
<td>0.35</td>
<td>0.38</td>
<td>0.34</td>
<td>-8.6</td>
</tr>
<tr>
<td></td>
<td>Protein-like</td>
<td>0.55</td>
<td>0.52</td>
<td>0.55</td>
<td>5.7</td>
</tr>
<tr>
<td>12 (salinity = 2.84‰)</td>
<td>Humic-like</td>
<td>0.11</td>
<td>0.11</td>
<td>0.11</td>
<td>-2.2</td>
</tr>
<tr>
<td></td>
<td>Fulvic-like</td>
<td>0.35</td>
<td>0.36</td>
<td>0.35</td>
<td>-1.7</td>
</tr>
<tr>
<td></td>
<td>Protein-like</td>
<td>0.45</td>
<td>0.42</td>
<td>0.44</td>
<td>5.9</td>
</tr>
</tbody>
</table>

<sup>a</sup>Model prediction: Calculated = (original measurement) – [slope × (salinity difference)]; also see text.

<sup>b</sup>Signal difference = (salt-adjusted – calculated)/calculated × 100.
Slow mixing of different types of waters at mid-pH (5.1–7.5 and 7.6–9.2) might not significantly affect the fluorescence signals of the NOM constituents.

This work also proposed a new empirical relationship between salinity and fluorescence components. We reported small intensity changes in the salinity range from ~0.02 to ~3‰. The signal change was between 2.7 and 3.4% per salinity unit (%). Interpretation of fluorescence data would still yield valid conclusions if, for example, saline deep waters typical of the CS mix or interact with shallow groundwaters (low ionic content).

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REFERENCES


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